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COAGULATION IN THE CRAYFISH, *ASTACUS LEPTODACTYLUS*: ATTEMPTS TO IDENTIFY A FIBRINOGEN-LIKE FACTOR IN THE HEMOLYMPH

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Occurrence of plasmatic clottable protein is one of the most controversial problems of decapod serology. Data are often inconsistent and vary according to the species under consideration. Previous electrophoresis (Durliat, 1974) carried out on crayfish serum and plasma show in the plasma the presence of an additional component which displays a role in coagulation, since it disappears after gelling of the blood. This additional fraction is relatable to the plasmatic clottable extract obtained by the procedures of Duchâteau and Florkin (1954) and Stewart, Dingle and Odense (1966). It is exciting to think that crayfish blood contains a fibrinogen-like protein analogous to that of *Panulirus interruptus* (Fuller and Doolittle, 1971a, b; Doolittle and Fuller, 1972; Tyler and Scheer, 1945), *Homarus americanus* (Stewart *et al.*, 1966) and *Homarus* sp. (Duchâteau and Florkin, 1954).

The work reported here is an attempt to isolate from *A. leptodactylus* hemolymph a plasma protein which participates in clotting and to determine some of its properties.

Materials and Methods

Serum and plasma preparation

Serum and plasma pools were obtained from 60 crayfish of both sexes, in a premolt stage (D0 to D3). Hemolymph was withdrawn from each crayfish's pereiopod sinus, then transferred into three tubes. Two samples were immediately and thoroughly mixed with an anticoagulant, a 10% sodium citrate or 0.1 M potassium oxalate solution. Proportions used were one part of anticoagulant for nine parts of hemolymph. This procedure prevented the clumping but not the breakdown of the cells. Plasma was collected after a 4° C centrifugation at 5000 rpm for 20 minutes. The precipitate was discarded and supernatant solution was retained. To obtain serum, hemolymph was allowed to form a nonretracting firm clot in a tube. *Astacus leptodactylus* is a decapod belonging to the coagulation C group (Tait and Gunn, 1918). In this clot, agglutination of hemocytes is insignificant but the gelling of plasma is the most important process. The clot was broken up with a stirring rod and centrifuged also at 5000 rpm. The supernatant was collected and the remaining coagulum discarded.

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However another method (Durliat and Vranckx, 1976) of preserving the hemolymph from clotting without damaging the cells gives the same results. This method follows that given by Tyson and Jenkin, 1974, with slight modifications. Crayfish were injected *via* the ventral haemal sinus with 2 ml of 0.25% cysteine hydrochloride in physiological saline containing 20 units of preservative heparin/ml at pH 6.2. After three minutes the animals were bled from the ventral sinus using a syringe containing 1 ml of 0.25% cysteine hydrochloride and 20 units of heparin/ml. The cysteine hydrochloride prevents the hemolymph from clotting, without damaging the cells. Heparin, while not preventing clotting, appears to prevent the clumping phenomenon. The contents of the syringe were, after removing the needle, gently emptied into an ice-cold tube and centrifuged to 3000 rpm for five minutes. Following centrifugation, the cells were resuspended in ice-cold van Harrevald's medium at pH 7.2 containing 20 units of heparin/ml. The supernatant or plasma without cells was retained.

Titration of hemolymph constituents

Measurements were made on Technicon. Total protein rate was detected by the method of Lowry, Rosebrough, Farr and Randall (1951), and amounts of triglycerids, phospholipids, cholesterol, uric acid and glucose of serum and plasma pools were determined.

Plasmatic clottable protein extractions

Salting out procedures, as used by Duchâteau and Florkin (1954) and then Stewart *et al.* (1966) on the blood of *Homarus* sp., were applied to freshly withdrawn pools of citrated or oxalated hemolymphs.

The clottable solution was dialysed against four changes of distilled water for 12 hours to eliminate SO_4 $(NH_4)_2$ which would inhibit the gelling, before setting up the coagulation tests.

Coagulation assays

These were performed on complete oxalated or citrated hemolymph (anticoagulant solutions were prepared in distilled water), on serum discarded from the gel fraction after coagulation and on "clottable protein" solutions obtained by the salting out method. Complete hemolymph (500 μl) is clotted with a mixture of 50 μl 0.1 M $CaCl_2$ and coagulable protein extract (200 μl) to which is added 50 μl 0.1 M $CaCl_2$ and 50 μl of cellular extract prepared by the method of Stewart *et al.* (1966).

Electrophoretic analysis

Disc electrophoresis was performed on 6% acrylamide gels according to the method of Ornstein (1964), with a tris glycine buffer pH 8.5 at 2 mAmp per tube for two hours on a concave polyacrylamide gel gradient (gradipore) with a tris borate buffer, with or without EDTA, at pH 8.2. Migrations were carried out for 24 hours at 60 V (15 mAmp). Proteins were stained by black amido 10 B during

one hour and faded by 7% acetic acid. The cupric fractions were stained by rubeanic acid (Decleir, 1961).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis procedures were essentially those described by Schwartz, Pizzo, Hill and McKee (1971), in continuous buffer. All preparations were incubated at 37° C for 24 hours in 0.04 M monodiphosphate buffer pH 7.1 containing urea 3% SDS, in the presence and absence of 3% mercaptoethanol. Gels were run in phosphate buffer containing 0.1% SDS for 19 hours at 15 mAmp and 30 V; they were stained with Coomassie blue after fixing in 10% acetic acid.

Immunologic analysis

Animal immunization. Antisera against serum, plasma and clottable protein were prepared in the I.R.S.C. of Villejuif, using for each solution two "Géant des Flandres" rabbits. They were inoculated with a pool from whole citrated hemolymphs of five crayfish of both sexes, according to the following method of Kabat modified by Burtin (personal communication).

Each rabbit received, in subcutaneous injections into the hind paws, a mixture of 0.5 ml antigens plus 0.5 ml Freund's adjuvant. Two weeks later, a series of three inoculations were effected during three consecutive days with 0.5 ml mixture containing: 0.5 ml antigens, 0.5 ml physiological saline and 0.5 ml 1% ammonium alum. The first injection was made subcutaneously, the next two intravenously. The products were tested, five days after the last inoculation, and the ultimate puncture was rechecked a week later. Antiplasma was obtained from a first pool of hemolymphs during the months of September through October, and antiserum from a second pool during the month of February.

Immunoelectrophoresis. Immunosera were tested by the double diffusion procedure (Ouchterlony, 1967) and immunoelectrophoresis with 1% agarose indubiose A37 gels. Techniques from Grabar and Burtin (1960), Laurell (1966) and Clarke and Freeman (1967) modified by Krøll (1973) were successfully employed; the migrations were carried out in 0.3 M tris barbital sodium barbital buffer pH 8.8. In the qualitative analysis, according to Grabar and Burtin's procedure, electrophoresis was run for 1.5 hr at 160 V and 15 mAmp; quantitative studies in Laurell required a 16 hour migration at 160 V and 18 mAmp and tandem-crossed immunoelectrophoreses were made in the first dimension for 25 hr at 160 V and 15 mAmp and during 18 hr with 130 V and 12 mAmp in the second dimension. Sera and plasmas were diluted by half. Pure solutions of plasmatic clottable extract were used.

Results

Titration of serum and plasma constituents

The results (Table I) are only available for the hemolymph of studied animals in premolt stages. A significant difference was noted between serum and plasma in the total protein content: 7 g liter. It might be argued that this divergence results from the mobilization of the coagulable plasmatic protein at the time of hemolymph gelling.

Electrophoretic analysis

Serum and plasma. Electrophoretic differences between serum and plasma from the same pool of hemolymphs were always observed. On 6% polyacrylamide gels, earlier experiments showed (in the plasma) an additional band (number 1) stopped at the cathodic part of the gel and another one (number 5) which is much more important than in the serum (Durliat, Vranckx, Herberts and Lachaise, 1975). On continuous gradient gels, the plasmatic clottable fraction might show both forms: either as a band of heavy molecular weight or as a multiple banding pattern (Fig. 1). These bands move into the gel approximately between the α_2 macroglobulin and the β lipoprotein position. Because of the logarithmic aspect of the banding pattern, it seems that these different fractions represent the polymeric states of a single subunit.

Specific determination of copper (rubeanic acid) shows identical cuproproteinaceous fractions in serum and plasma. Therefore the clottable factor which disappears or is very faint in the serum probably represents a protein displaying a role in the coagulation processes.

Plasmatic clottable protein. In the same conditions, electrophoresis of different fractions precipitated by ammonium sulfate, showed that the second precipitate (45% saturation) effectively presented an important enrichment in a component corresponding either to the supplementary plasmatic band or to the multiple banding pattern and shadows of the other fractions. The impurities can be partly eliminated after filtration on an agarose A 1.5 column. It was noted that the first precipitate obtained by the salting out procedure (30% saturation) also contained small quantities of clottable protein.

SDS electrophoresis (Fig. 2). Differences between serum and plasma persisted after incubation with SDS, which abolishes all variations proceeding from electrical charges. The clottable extract occurred as a fraction in the cathodic position.

Clotting studies

Whole hemolymph. One ml of whole oxalated hemolymph with the addition of 100 μl 0.1 M calcium chloride clotted very strongly in 15 minutes. The assays performed on 37 animals were all successful. However, when the same experiments were performed on citrated hemolymph, using sodium citrate solution pre-

TABLE 1

Titration of serum and plasma constituents.

	Serum g/liter	Plasma g/liter
Total protein content	39.5	46.4
Triglycerids	0.20	0.22
Phospholipids	0.80	0.88
Cholesterol	0.40	0.40
Uric acid	0.20	0.20
Glucose	0.45	0.45

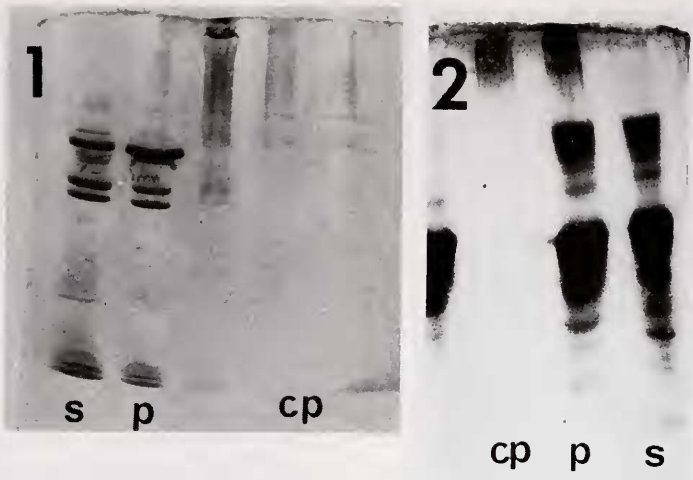


FIGURE 1. Electrophoresis on continuous gradient gels of the crayfish hemolymph, in tris borate EDTA buffer pH 8.2. Serum is represented by (s); plasma (p); and clottable protein (cp).

FIGURE 2. Sodium dodecyl sulfate electrophoresis after incubation in SDS and urea presence. Gel was run in phosphate buffer containing 0.1% SDS for 15 hours with 15 mAmp and 30 V. Symbols are as in Figure 1.

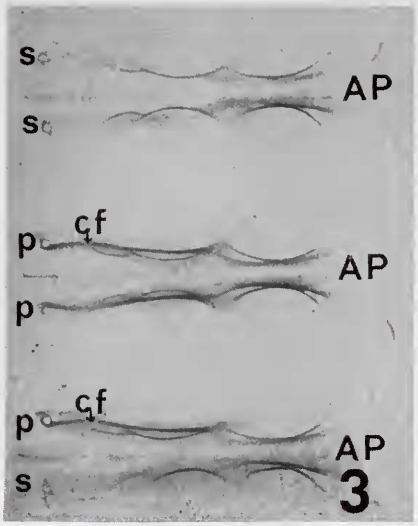
pared in distilled water, no clot was formed. It seems that this anti-coagulant chelates the calcium necessary for the transformation of the clottable protein into gel and also irreversibly blocks the conversion. The solutions of sodium citrate or potassium oxalate do not block the hemolymph clotting in the same way. With sodium citrate, univalent ions were necessary to permit the reversibility of this reaction. It was noted that the pH values of sodium citrate in distilled water or in physiological saline were almost identical.

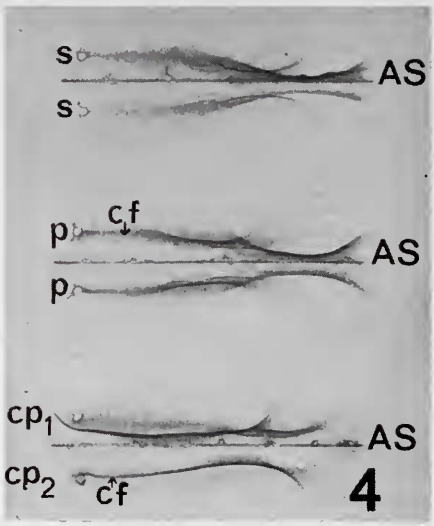
However when univalent ions were added in the citrate solution, before the mixture with hemolymph, the clotting appeared easily (0.5 ml of blood was withdrawn on 100 μl 0.1 M sodium citrate + 100 μl 0.1 M NaCl or KCl and clotted in 15 minutes following the addition of 100 μl 0.1 M $CaCl_2$).

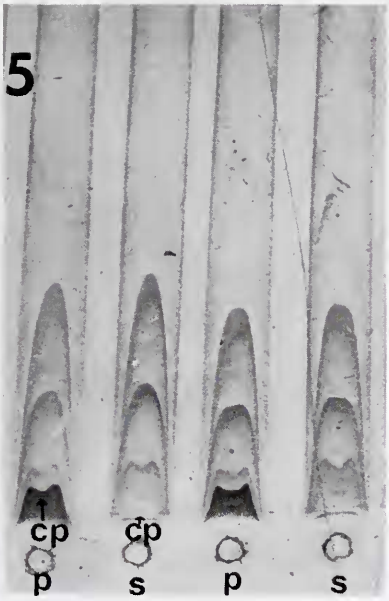
Plasmatic clottable protein. When obtained from oxalated plasma, it clotted very strongly within 10 minutes (0.1 ml clottable fraction + 50 μl 0.1 M $CaCl_2$ + 50 μl cellular extract); but it failed to elicit a clotting response when it was extracted from citrated blood, by the same salting out procedure. However, with a citrated plasma containing Na^+Cl^- ions, the extraction furnished a solution which clotted perfectly.

On the polyacrylamide gradient gels, no differences were noted between the electrophoretic patterns of active and inactive preparations. Clotting tests performed with clottable protein reconstituted after storage in $(NH_4)_2\ SO_4$ were uniformly negative, but freezing seems to have no effect on its clottability.

Serum. The same assays were carried out with the serum to verify the presence or absence of coagulable proteins still available. Reclotting was not possible.







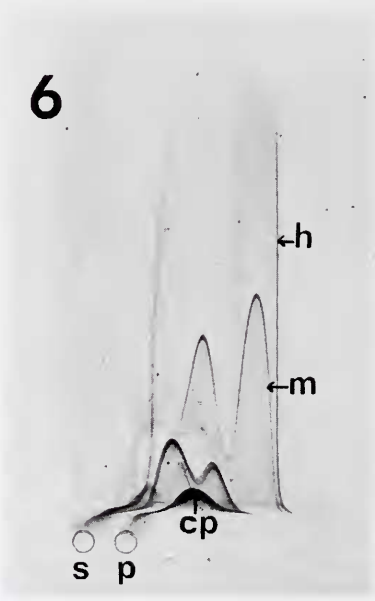


FIGURE 3. Immunoelectrophoresis against an antiplasma of crayfish. Migration for 1.5 hr with 160 V and 15 mAmp in tris barbital sodium barbital buffer pH 8.8. AP shows antiplasma; cf represents the precipitate line of clottable factor. Other symbols are as in Figure 1.

FIGURE 4. Immunoelectrophoresis against an antiserum of crayfish. Migration for 1.5 hr with 160 V and 15 mAmp in tris barbital sodium barbital buffer pH 8.8. Clottable protein extract before filtration (cp1) and after filtration (cp2) is shown on agarose column; AS shows antiserum. Other symbols are as in Figure 1 and Figure 3.

FIGURE 5. Immunoelectrophoresis according to Laurell. Symbols are as in Figure 1; and the antigens result from amounts of plasma and serum in each series from left to right

Immunologic analysis

When serum and plasma were tested against an antiplasma, a distinct precipitate line was present in the plasma (Fig. 3), but appeared only as a shadow in the serum. Clottable extract showed the same precipitate line when tested against this antiplasma. It was noted that this isolated component was not exempt from minor contaminants, because two other arcs were present. One of these other fractions appeared to be hemocyanin. By varying the antigen/antibody ratio, and especially in a large excess of antibody, it was possible to obtain the same picture against an antiserum (Fig. 4).

By quantitative immunoelectrophoresis (Fig. 5) both serum and plasma gave the same number of peaks. When a protein is present in the same amounts in samples, peaks of the same height are observed. This was the case with all but one of the proteins of both serum and plasma. On Figure 5 the lowest peak, assumed to be the clottable factor, was well represented in the plasma but was insignificant in the serum.

Immunological identities between the different proteins of both serum and plasma were evidenced by tandem-crossed immunoelectrophoresis (Clarke and Freeman, 1967). All proteins gave double-headed peaks except the clottable factor (Fig. 6).

Furthermore, by Ouchterlony diffusions, a protein with a complete antigenic identity appeared in the serum, plasma and clottable fraction (Fig. 7). There is still clottable protein in the serum because a single clotting process does not remove all the plasmatic coagulable protein. This phenomenon was clarified in the spiny lobster *Jasus paulensis,* in which serum was reclotted several times (Durliat and Vranckx, in preparation). The reclotting of *Astacus* serum was not possible because the amount of the remaining clottable protein was too low. This protein disappeared in the sample of serum when it was absorbed with an anti-clottable extract.

Discussion

Evidenced in *Homarus* sp. (Duchâteau and Florkin, 1954), *Homarus americanus* (Stewart *et al.,* 1966), *Callinectes sapidus* and some other decapods (Manwell and Baker, 1963), and *Panulirus interruptus* (Fuller and Doolittle, 1971 a, b; Doolittle and Fuller, 1972), a plasmatic "fibrinogen"-like factor has not been found in *Gecarcinus lateralis* (Stutman and Dolliver, 1968), *Cancer irroratus, Cancer*

of 30 μg and 25 μg. For the antibody an agarose gel containing 1% rabbit serum anticrayfish plasma (2 μl/cm²) is used. Migration run for 16 hours with 160 V and 18 mAmp in tris barbital sodium barbital buffer pH 8.8 at 4° C. Note that the same number of peaks occur in serum and plasma, but that the clottable factor very important in plasma was insignificant in serum.

Figure 6. Tandem crossed immunoelectrophoresis. Premolt protein is represented by m; hemocyanin by h. Other symbols are as in Figure 1. An antibody of agarose gel containing 6% rabbit serum anticrayfish plasma (10 μl/cm²) is used. Antigens involve amounts of serum and plasma of 100 μg. Migrations run in first dimension: 2.5 hr with 160 V and 15 mAmp; second dimension: 18 hours with 130 V and 12 mAmp, in tris barbital sodium barbital buffer pH, 8.8 at 4° C. All proteins give double-headed peaks, except the clottable factor.

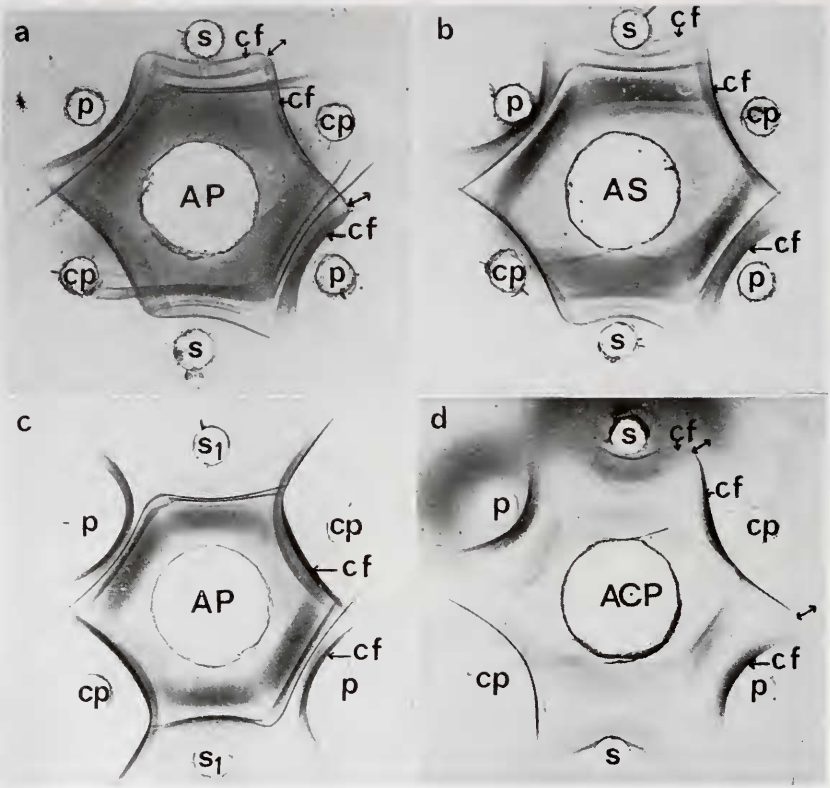


FIGURE 7. Ouchterlony studies with as antibodies: antiserum (100 μl) represented by AS; antiplasma (100 μl) by AP; and anticlottable protein (2.5 μl) by ACP. For the antigens: serum absorbed with anticlottable protein is represented by S1; precipitate line of clottable factor by cf. Other symbols are as in Figure 1. The clottable factor of serum, plasma and coagulable extract gives a continuous precipitate line showing a complete identity (↔). In Figure 7c this precipitate line is not evidenced against S1 because remaining clottable factor was absorbed against anticoagulable protein. On each immunodiffusion, two different amounts of S, P and cp are loaded so that variation in density of precipitate line are recorded.

borealis, Hyas coarctatcus (Stewart and Dingle, 1968), nor *Oronectes virilis* (Wood and Karpawich, 1972). More exactly, it was not detected by electrophoretic analysis. In the last cases plasma and serum proteins were, according to these authors, almost identical.

It is possible that the explanation of this difference lies in the quantities of available plasmatic clottable protein which, when these quantities are too low, cannot be detected by electrophoretic analysis. For instance, in *Gecarcinus lateralis,* plasma and serum electrophoregrams are similar, but hemolymph microscopic observations show the development of fibrin-like strands (Stutman and Dolliver, 1968).

Moreover, other observations show that the level of plasmatic clottable factor depends not only on the species of the examined animal, but also on its physiological state (intermolt stage, captivity, pathology). Therefore, it is likely that the con-

junction of all these factors, internal and external, may explain the great variability of data already reported in the literature on plasmatic clottable protein.

The heterogeneous aspect of this coagulable plasmatic protein was noted; it occurs either as a fraction of molecular weight 3,200,000 or as a multiple banding pattern. However in *Panulirus interruptus*, Doolittle and Fuller (1972) have revealed with SDS and mercaptoethanol electrophoresis that the clottable factor appears as a monomer unit with a molecular weight of 400,000 and a dimer of 800,000. Other observations (Durliat *et al.*, 1975) seem to indicate that in *Astacus leptodactylus* the clottable factor undergoes a polymerization as time goes on.

Our recent experiments have demonstrated that the monomer has its origin in the hemocytes. This protein is also present as an integral part of the plasma before any leakage from blood cells (Durliat and Vranckx, 1976). This clotting process seems to be different from that in the horseshoe crab *Limulus polyphemus*, which consists only in the conversion of a clottable protein located entirely in the hemocytes into a gel by an enzymatic system (Levin and Bang, 1968; Young, Levin and Prendergast, 1972).

On the other hand, lacking knowledge of the structure of this protein, presence of traces of clottable-related fractions in the serum may be explained either as a fibrinopeptid resulting from degradation of the native protein or as the rest of this gelling protein which has not clotted. However, Fuller and Doolittle (1971b) show an absence of the "fibrinolysis" process as it occurs in the vertebrates. In our work, Ouchterlony diffusions do not seem to show any difference between the clottable fractions of various origins. One may postulate that during coagulation all the clottable fractions are not needed nor used and that the remainder found in the serum may still induce specific antibody formations in the rabbit. This explains the fact that the same kinds of immunoelectrophoresis are observed when plasma is tested against either antiplasma or antiserum. Thus, these antibodies exist together in the antiserum and antiplasma, but the precipitation reaction is obvious only when the stoichiometric ratio is suitable.

Ionic requirements are only reported but not at all understood. It was established by reclotting assays that monovalent cation was needed to be added to the citrated solution before mixing with the hemolymph rather than to the oxalated solution. Is the clottable protein or the clotting enzyme irreversibly disturbed by the citrate in the absence of a monovalent cation?

On the other hand, the tandem-crossed immunoelectrophoresis carried out against an antiplasma (Fig. 6, m) shows a protein existing in serum and plasma, but disappearing when the samples are tested against an antiserum. Antiplasma has been prepared in September from hemolymphs of premolt stage animals and antiserum in February from crayfish blood in intermolt (C4); one can postulate that it is a cuticular protein becoming overt in the crayfish approaching the molt. This supposition has been confirmed with antiserum and antiplasma obtained from same hemolymph pool from animals in different intermolt stages (Vranckx and Durliat, 1976).

In summary, the tests performed on the hemolymph of *Astacus leptodactylus* demonstrate a coagulable plasmatic protein, analogous to a "fibrinogen"-like fraction, of large molecular weight and high antigenicity.

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Summary

1. A series of tests were conducted to determine whether or not the hemolymph of the crayfish *Astacus leptodactylus* contains a plasma coagulation factor.

2. The total protein amount is higher in the plasma than in the serum.

3. Serum and plasma do not exhibit similar electrophoretic banding patterns. Plasma contains one band or a series of supplementary fractions with a high molecular weight.

4. Electrophoregrams of plasmatic clottable extract, obtained by the classical methods employed in crustacean serology, show a main fraction or a series of polymers with the same electrophoretic behavior as the additional fractions seen on the plasma pattern.

5. This solution clots when treated with $CaCl_2$ and a cellular extract.

6. Immunoelectrophoresis demonstrates the presence of a clottable protein precipitate line in plasma, but this protein also gives a very faint similar line in serum.

LITERATURE CITED

Clarke, H. G. M., and T. Freeman, 1967. A quantitative immunoelectrophoresis method (Laurell electrophoresis). *Prot. Biol. Fluids,* **14**: 503–509.

Decleir, W., 1961. The localization of copper in agar gel electrophoresis patterns of crustacean blood. *Naturwissenschaften,* **48**: 102–103.

Doolittle, R. F., and G. M. Fuller, 1972. Sodium dodecyl sulfate polyacrylamide gel electrophoresis studies on lobster fibrinogen and fibrin. *Biochem. Biophys. Acta,* **263**: 805–809.

Duchâteau, G., and M. Florkin, 1954. La coagulation du sang des Arthropodes. IV. Sur le fibrinogène et sur la "coaguline" musculaire du Homard. *Bull. Soc. Chim. Biol.,* **36**: 295–305.

Durliat, M., 1974. Etude de la coagulation de l'hémolymphe chez les Crustacés Décapodes. Analyse électrophorétique des protéines du sérum et du plasma d'Ecrevisse (*Astacus leptodactylus*). *C. R. Hebd. Séanc. Acad. Sci. Paris Ser. D,* **278**: 473–476.

Durliat, M., and R. Vranckx, 1976. Analyse des protéines hémocytaires d'*Astracus leptodactylus*. *C. R. Hebd. Séanc. Acad. Sci. Paris Sér. D,* in press.

Durliat, M., R. Vranckx, C. Herberts, and F. Lachaise, 1975. Effets de la congélation sur la séparation electrophorétique des protéines de l'hémolymphe de quelques Arthropodes. *C. R. Séanc Soc. Biol.,* **169**: 862–867.

Fuller, G. M., and R. F. Doolittle, 1971a. Studies of invertebrate fibrinogen. I. Purification and characterization of fibrinogen from spiny lobster. *Biochemistry,* **10**: 1305–1310.

Fuller, G. M., and R. F. Doolittle, 1971b. Studies of invertebrate fibrinogen. II. Transformation of lobster fibrinogen into fibrin. *Biochemistry,* **10**: 1311–1315.

Grabar, P., and P. Burtin, 1960. Analyse immuno-électrophorétique. Ses applications aux liquides biologiques humains. Masson et Cie, Paris.

Krøll, J., 1973. Quantitative immunoelectrophoresis. 4. Tandem crossed immunoelectrophoresis. *Scand. J. Immunology, Suppl.,* **1**: 57–59.

Laurell, C. B., 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.,* **15**: 45–52.

Levin, J., and F. B. Bang, 1968. Clottable protein in *Limulus:* its localization and the kinetics of its coagulation by endotoxin. *Thromb. Diath. Heamorrh.,* **19**: 186-197.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265–275.

Manwell, C., and C. M. A. Baker, 1963. Starch gel electrophoresis of sera from some marine Arthropods: studies on the heterogeneity of hemocyanin and on a "ceruloplasmin like protein." *Comp. Biochem. Physiol.*, **8**: 193–208.

Ornstein, L., 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.*, **121**: 321–349.

Ouchterlony, O., 1967. Immunodiffusion and immunoelectrophoresis. Pages 655–706 in D. M. Weir, Ed., *Handbook of Experimental Immunology*. Blackwell, Oxford and Edinburgh.

Schwartz, M. L., S. V. Pizzo, R. L. Hill, and P. A. McKee, 1971. The effect of fibrin-stabilizing factor on the subunit structure of human fibrin. *J. Clin. Invest.*, **50**: 1506–1513.

Stewart, J. E., and J. R. Dingle, 1968. Characteristics of hemolymph of *Cancer irroratus, Cancer borealis* and *Hyas coarctatus*. *J. Fish. Res. Board Can.*, **25**: 607–610.

Stewart, J. E., J. R. Dingle, and P. H. Odense, 1966. Constituents of the hemolymph of the lobster *Homarus americanus* Milne Edwards. *Can. J. Biochem.*, **44**: 1447–1459.

Stutman, L. J., and M. Dolliver, 1969. Mechanism of coagulation in *Gecarcinus lateralis*. *Ann. Zool.*, **8**: 481–489.

Tait, J., and D. J. Gunn, 1918. The blood of *Astacus fluviatilis:* a study in crustacean blood, with special reference to coagulation and phagocytosis. *Quart. J. Exp. Physiol.*, **12**: 35 80.

Tyler, A., and B. T. Scheer, 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus*. II. Chemical and antigenic relation to blood proteins. *Biol. Bull.*, **89**: 193–200.

Tyson, C. J., and C. R. Jenkin, 1974. Phagocytosis of bacteria *in vitro* by haemocytes from the crayfish (*Parachaeraps bicarinatus*). *Aust. J. Exp. Biol. Med. Sci.*, **52**: 341–348.

Vranckx, R., and M. Durliat, 1976. Analyse immunologique des variations des protéines de l'hémolymphe d'*Astacus leptodactylus* pendant la prémue. *C. R. Hebd. Séanc. Acad. Sci. Paris Ser. D.*, 282: 1305-1308.

Wood, P. J., and P. P. Karpawich, 1972. Studies of the coagulation process in the crayfish, *Orconectes virilis:* attempts to identify a fibrinogen-like factor. *Comp. Biochem. Physiol.*, **42**: 41–48.

Young, N. S., J. Levin, and R. A. Prendergast, 1972. An invertebrate coagulation system activated by endotoxin. Evidence for enzymatic mediation. *J. Clin. Invest.*, **51**: 1790.